Polymorphisms in Cytoplasmic Serine Hydroxymethyltransferase and Methylenetetrahydrofolate Reductase Affect the Risk of Cardiovascular Disease in Men[1]

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ABSTRACT Genetic variation in folate-regulating enzymes contributes to the risk of cardiovascular disease (CVD). The cytoplasmic serine hydroxymethyltransferase (cSHMT) enzyme is proposed to regulate a key metabolic intersection in folate metabolism. We hypothesized that a variant in *cSHMT* (*cSHMT* 1420C→T) affects CVD risk, and that the effect depends on a linked step in the metabolic pathway catalyzed by methylenetetrahydrofolate reductase (MTHFR). A nested case-control study of incident CVD was conducted within the all-male Normative Aging Study cohort. Of the incident CVD cases, 507 had DNA samples; 2 controls/case were selected by risk set sampling (matched on age and birth year). A significant gene-gene interaction (*P*-values 0.0013, 0.0064) was found between *MTHFR* and *cSHMT*, and there was little or no change in the coefficients in covariate-adjusted models. The effect of *MTHFR* 677C→T genotype on CVD risk varied by *cSHMT* 1420C→T genotype. Among men with *cSHMT* 1420C→T *TT* genotype, the odds ratios (OR) for CVD risk for *MTHFR* 677C→T *CT* and *TT* genotypes compared with the *MTHFR* 677C→T *CC* genotype were 3.6 (95% CI, 1.7–7.8) and 10.6 (95% CI, 2.5–46.0), respectively. Among men with the *cSHMT* 1420C→T *CC/CT* genotype, the corresponding ORs were 1.0 (95% CI, 0.8–1.2) and 1.3 (95% CI, 0.9–1.8). Plasma total homocysteine concentrations were highest in the subgroup of men with both polymorphisms, *MTHFR* 677C→T *TT* and *cSHMT* 1420C→T *TT*, consistent with a higher risk of CVD in this subgroup. A more complete understanding of the molecular mechanism awaits identification of the functional effect of the polymorphism. J. Nutr. 135: 1989–1994, 2005.

KEY WORDS: • *homocysteine* • *folate* • *men* • *cardiovascular disease*

The importance of folate in health is well established on the basis of its critical role in metabolism and its relevance to public health even in well-nourished Western populations (1). Folate cofactors carry 1-carbon units that are essential for nucleotide biosynthesis and numerous methylation reactions (2). Altered metabolism of folate is related to the pathogenesis of neural tube defects, cancer, and cardiovascular disease (CVD)[3] (3).

Cytoplasmic serine hydroxymethyltransferase (cSHMT) is a key enzyme affecting the intracellular homeostasis among folate cofactors (4). cSHMT reversibly converts serine and tetrahydrofolate (THF) to glycine and 5,10-methylenetetrahydrofolate (5,10-methyleneTHF) (4). The 1-carbon moiety of 5,10-methyleneTHF is in turn directed to the synthesis of purines or thymidylate or to the methionine cycle where it remethylates homocysteine to synthesize methionine and *S*-adenosyl methionine (2). Three functions of cSHMT are indicated: isotope tracer studies suggest that cSHMT preferentially supplies 1-carbon units to thymidylate biosynthesis; cSHMT catalyzes glycine-dependent serine synthesis, hence

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[3] Abbreviations used: CC, genotype refers to either MTHFR 677 CC or cSHMT 1420 CC; cSHMT, cytoplasmic serine hydroxymethyltransferase; CVD, cardiovascular disease; 5-methylTHF, 5-methyltetrahydrofolate; 5,10-methyleneTHF, 5,10-methylenetetrahydrofolate; MTHFR, methylenetetrahydrofolate reductase; NAS, Normative Aging Study; OR, odds ratio; SNP, single nucleotide polymorphism; THF, tetrahydrofolate; TT, genotype refers to either MTHFR 677 TT or cSHMT 1420 TT.

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depleting 5,10-methyleneTHF for *S*-adenosyl methionine synthesis; and cSHMT sequesters 5-methyltetrahydrofolate (5-methylTHF), hence decreasing *S*-adenosyl methionine synthesis (5).

A single nucleotide polymorphism (SNP) in the *cSHMT* gene was identified: the base at nucleotide 1420 is either C or T, resulting in the amino acid leucine or phenylalanine, respectively, at position 474 of the protein (6). Screening of the coding and regulatory regions of the *cSHMT* gene yielded only 2 other variants: *cSHMT* 1181G→A, an extremely rare variant (6), and an E to Q substitution at amino acid position 340 (7).

Direct evidence regarding the functional consequence of the *cSHMT* 1420C→T polymorphism is not available, but there is some evidence that the SNP is associated with both metabolic disruption and disease risk. Heil et al. (6) reported lower homocysteine concentrations among mothers whose children had neural tube defects when the mothers were homozygous for the 1420C→T variant of *cSHMT* (*TT*) (compared with mothers with the CC genotype). In another group of unselected individuals, there were no effects on homocysteine concentrations; neither of these findings was stratified for methylenetetrahydrofolate reductase (*MTHFR*) variants (6). A recent study found both the *CT* and the *TT* genotypes of *cSHMT* associated with a substantially reduced risk of leukemia relative to the *cSHMT* 1420C→T CC genotype (8). A study of colorectal cancer risk reported no association of the *cSHMT* SNP with either homocysteine concentration or with cancer risk (9).

This study was designed to test the hypothesis that the *cSHMT* 1420C→T variant affects the risk of CVD. Because polymorphisms in the *MTHFR* gene, particularly *MTHFR* 677C→T (10,11), affect functionality of a linked step in the folate metabolic network, we hypothesized that the relation of *cSHMT* 1420C→T to CVD risk would differ according to *MTHFR* genotype. This study explored whether the genotype-disease association was consistent with the effects of the genotype on plasma total homocysteine concentration, a biological marker of folate-dependent methylation, which was measured in a subset of cases and controls. The role of dietary intake of folate and other B vitamins on the genotype-disease association was also explored.

SUBJECTS AND METHODS

Study population. A nested case-control study was conducted using the prospective Normative Aging Study (NAS) cohort. The NAS was established by the Veterans' Administration in 1961, and 2280 men aged 21–81 (mean age of 42 y at study entry) were selected on the basis of health criteria (12). Men with past or current chronic conditions, including coronary heart disease, hypertension, diabetes, cancer, peptic ulcer, gout, asthma, chronic bronchitis, and chronic sinusitis were ineligible. Since the time of enrollment, participants have had comprehensive clinical examinations at 3- to 5-y intervals with a response rate > 90% for mailed questionnaires, including FFQs. As of June 1998, 543 participants (24%) were deceased and ~1600 men (70%) were actively participating in follow-up visits (mean age of 70 y). The rate of continued participation of NAS men over the follow-up period was excellent, with <1% annual attrition for all causes.

From November 1961 through December 1998, 749 incident cases of coronary heart disease and stroke occurred. Possible occurrences of nonfatal coronary heart disease, including angina pectoris and myocardial infarction, were evaluated based on medical records and physician examination (13). Angina was defined by Framingham Heart Study criteria as recurrent chest discomfort lasting up to 15 min distinctly related to exertion or excitement that was relieved by rest or nitroglycerin (13). Nonfatal myocardial infarction was diagnosed only when documented by unequivocal electrocardiography changes and chest discomfort consistent with myocardial infarction. Possible occurrences of nonfatal stroke were identified by report of a neurological deficit of sudden or rapid onset that persisted for 24 h or longer, and all reports were confirmed by a neurologist's review of the medical records (14). Occurrences of fatal coronary heart disease or stroke (in men with no prior nonfatal CVD event) were confirmed by death certificates, which were coded according to the 8th revision of the International Classification of Diseases (15); ~6% of cases had only stroke. DNA was available for 72% of cases; thus 535 incident CVD cases were studied. Cases without DNA samples were primarily cases that occurred early in the follow-up. The cases without DNA samples were older, had slightly higher systolic blood pressure, and had greater cumulative smoking exposure at the beginning of the cohort study.

Matched controls ($n = 1048$) were selected by risk-set sampling (16–18) and were matched to each case by age at onset and birth period (in 5-y intervals) of the case. Thus, as each case occurred, all other cohort members at risk (including cases that occurred at later times), under active follow-up, with the same matching conditions, and with available DNA formed a risk set for the incident case. Two men from the risk set were randomly chosen as matched controls. Cases were eligible to be a control in the time period before they were diagnosed, and some men were chosen as a control for more than one case (19). The study was approved by the Brigham and Women's Hospital Human Subjects committee, the Veterans' Administration R & D committee, and the Cornell University Committee on Human Subjects.

Data collection: covariates. Extensive data are available on these men, including physical measurements, lifestyle factors, and some blood analyses. Beginning in 1987, men completed an FFQ referring to intake in the prior year (20), at every visit. Estimates of dietary intake, including B vitamins, methionine, coffee, and alcohol, were derived from the frequency and dosage information on the FFQ using software developed by the Nurses' Health Study (20). At least 1 FFQ was available before the date of diagnosis for about half of the CVD cases and noncases. Serum HDL cholesterol was measured beginning in 1981, but the majority of cases had HDL measurements before the CVD event occurred. Lipids were assayed over the course of the follow-up as follows: serum cholesterol was assayed enzymatically (SCALVO Diagnostics); HDL cholesterol was measured in the supernatant after precipitation of the LDL cholesterol and VLDL fractions with dextran sulfate and magnesium, using the Abbott Biochromatic Analyzer 100 (Abbott Laboratories); triglyceride was measured with a Dupont ACA discrete clinical analyzer (Biomedical Products Department, Dupont). Plasma total homocysteine was assayed in an unselected subset of stored blood samples. Plasma samples were stored at −80°C, and transferred to the Jean Mayer USDA Human Nutrition Research Center on Aging, where they were analyzed. Total homocysteine in plasma was determined by an adaptation of the method described by Araki and Sako (21). The CV for this assay was 4.0%. Homocysteine data were available for ~54% of cases and 72% of controls. Most (93%) of the plasma homocysteine data were obtained from blood samples collected after the CVD event.

Data collection: genotyping methods. In 1999, DNA was extracted from stored frozen buffy coat of 7 mL of whole blood, using the QiAmp DNA blood kits (QIAGEN). Genotypes of 3 polymorphisms (*cSHMT* 1420C→T, *MTHFR* 677C→T, and *MTHFR* 1298A→C) were determined by the TaqMan procedure using the allelic discrimination technique (ABI Prism 7900 Sequence Detection System, Applied Biosystems). The DNA samples for cases and controls were plated in random order with a mixture of TaqMan Universal PCR Master Mix, primers, and probes. PCR cycling conditions consisted of one 2-min cycle at 50°C, one 10-min cycle at 95°C, followed by 40–46 cycles at 95°C for 15 s and at 60°C for 1 min. The methods for the analysis of the *cSHMT* 1420C→T polymorphism were described in detail elsewhere (8), and the primers and probes for the 2 *MTHFR* polymorphisms were created according to standard methods for the TaqMan procedure. Genotypes were successfully determined for 96% of the study group.

Statistical analysis. Genotype frequencies among controls were compared with those expected in Hardy-Weinberg equilibrium (22)

TABLE 1

Characteristics at study entry of cases and their matched controls, Normative Aging Study, 1961–1998[1]

Variable	Cases	Matched controls
Diastolic blood pressure, *mm Hg*	77.7 ± 8.4	76.4 ± 8.4
Systolic blood pressure, *mm Hg*	125.0 ± 13.1	121.4 ± 12.2
Serum total cholesterol,[2] *mg/dL*	211.2 ± 45.3	198.8 ± 43.0
Serum HDL cholesterol,[2] *mg/dL*	45.0 ± 12.2	48.3 ± 13.3
Plasma total homocysteine, *μmol/L*	10.9 ± 3.2	10.4 ± 3.8
BMI, kg/m^2	26.3 ± 2.8	25.7 ± 2.9
Drinkers, usually ≥2/d, %	13.1	11.4
Current smokers, %	33.4	29.1
Duration of smoking, *y*	14.8 ± 12.8	13.0 ± 11.8
Cumulative smoking, *pack-y*	19.8 ± 21.4	15.9 ± 18.9
Vitamin intake		
Folate, *μg/d*	423 ± 255	427 ± 221
Vitamin B-6, *mg/d*	3.4 ± 5.6	3.4 ± 6.3
Vitamin B-12, *μg/d*	9.0 ± 6.4	9.8 ± 7.8

[1] Values are means ± SD or %, $n = 1304$. For a few variables, some data are missing ($n = 545$ minimum).
[2] To convert to SI units, multiply mg/dL by 0.0259 to compute mmol/L.

and tested with the χ^2 statistic. Linkage disequilibrium among the 3 polymorphic sites was tested using the Likelihood Ratio Test and Fisher's Exact Test (22). Ordinary least-squares regression models were used to estimate the relation of genotype to plasma total homocysteine, considering homocysteine as the outcome variable. Univariate procedures were used to estimate the covariate means for cases and their matched controls to identify potentially confounding factors. Conditional logistic regression analysis (SAS PHREG; SAS Institute) was used to analyze the risk set sampled cases and controls (19). To test whether the *cSHMT*-CVD association varied by other genotypes and to assess effect modification of the *cSHMT*-CVD association by other risk factors, product terms were included in the regression model.

The *cSHMT*-CVD association was estimated in unadjusted models and in models adjusted for major risk factors for CVD (covariates available on majority of cases and controls). In addition, the *cSHMT*-CVD association was estimated in the subset of cases and controls with dietary data, and in the subset with homocysteine data. Once again, further models were considered to assess potential confounding factors, mediating effects, and effect modification. Data are reported as regression coefficients or means and SE, and as odds ratios (ORs) with 95% CI. Differences were considered significant at $P < 0.05$, but exact *P*-values are shown for all comparisons.

RESULTS

The difference in the pattern of cardiovascular risk factors between cases and their matched controls (**Table 1**) was consistent, with greater CVD risk in cases; the possible influence of these covariates on the genotype-CVD association was accounted for in adjusted models. Table 1 provides a description of the characteristics of this all-male cohort, comprised of Caucasian men with a mean age of 42 y at the study baseline in 1961.

Allele frequencies for the polymorphic sites were considered in controls (**Table 2**) and were similar to past reports for *cSHMT* 1420C→T (11% *TT*) (6,8), *MTHFR* 677C→T (11% *TT*) (23), and *MTHFR* 1298A→C (10% *CC*) (24) in Caucasians. All 3 polymorphisms were in Hardy-Weinberg equilibrium, *MTHFR* 677C→T and *MTHFR* 1298A→C were in linkage disequilibrium, and neither *MTHFR* variant was in linkage disequilibrium with *cSHMT* 1420C→T.

There was little or no relation of the *cSHMT* 1420C→T *CT* genotype with CVD risk (vs. CC), both overall and stratified by the *MTHFR* 677C→T genotype. All further analyses therefore considered men with the *cSHMT* 1420C→T *CC* and *CT* genotype as a single group. The *MTHFR* 1298A→C genotype had little or no association with CVD risk, and there was no evidence of an interaction with the other genotypes; thus, this genotype was not considered further.

In unadjusted models to assess the main effect of *cSHMT* before considering other genotypes, there was a protective effect of small magnitude for the *cSHMT* 1420C→T *TT* genotype (OR 0.9; 95% CI 0.6–1.3). In models testing whether the *MTHFR*-CVD association differed by the *cSHMT* 1420C→T polymorphism, the gene-gene interaction was significant (*P*-values ranged from 0.0013 to 0.03 for model coefficients for interaction in **Table 3**). In the *cSHMT* 1420C→T *TT* genotype stratum, men with the *MTHFR* 677C→T *TT* genotype had ~10 times the rate of CVD vs. *MTHFR* 677C→T *CC*, and men with the *MTHFR* 677C→T *CT* genotype (vs. CC) had ~4 times the rate of CVD (**Table 4**). In the *cSHMT* 1420C→T *CC/CT* genotype strata, men with the *MTHFR* 677C→T *TT* genotype had an increase of ~30% in the rate of CVD vs. *MTHFR* 677C→T *CC*, and men with

TABLE 2

Cross-classification of MTHFR 677C→T, MTHFR 1298A→C, and cSHMT 1420C→T genotypes in control group, Normative Aging Study, 1961–1998[1]

	MTHFR 677 CC MTHFR 1298A→C			MTHFR 677 CT MTHFR 1298A→C			MTHFR 677 TT MTHFR 1298A→C			
	AA	AC	CC	AA	AC	CC	AA	AC	CC	Total
	n									*n (%)*
CC[2]	34	45	26	61	61	1	27	0	0	255 (48)
CT	29	44	17	51	46	0	26	1	0	214 (41)
TT	10	18	6	10	11	0	3	0	0	58 (11)
Total, *n (%)*		229 (43)			241 (46)			57 (11)		527

[1] Values are *n* and *n* (%) of controls ($n = 527$) with the genotype.
[2] cSHMT 1420C→T genotypes are CC, CT, TT, respectively.

TABLE 3

Multivariate models evaluating cSHMT 1420C→T, MTHFR 677C→T genotypes, and their interaction in relation to CVD risk, Normative Aging Study, 1961–1998

	Crude models[1]			Adjusted models[2]		
Model variable	β	SE	P	β	SE	P
cSHMT 1420C→T (TT vs. CC/CT)	−0.84091	0.3083	0.0064	−1.49804	0.5512	0.0066
MTHFR 677C→T (CT vs. CC)	−0.03018	0.1264	0.811	−0.24664	0.1852	0.1828
MTHFR 677C→T (TT vs. CC)	0.25930	0.1765	0.1418	−0.01132	0.2414	0.9626
Interaction, cSHMT 1420 and MTHFR 677 (CT vs. CC)	1.31936	0.4099	0.0013	1.87016	0.6656	0.0050
Interaction, cSHMT 1420 and MTHFR 677 (TT vs. CC)	2.10375	0.7721	0.0064	2.39283	1.1039	0.0302

[1] Values are regression coefficients, SE of regression coefficients, and *P*-values from crude (unadjusted) models, $n = 1514$ matched analysis.
[2] Values are regression coefficients, SE of regression coefficients, and *P*-values from models adjusted for homocysteine, $n = 987$ matched analysis, due to missing data on homocysteine.

the *MTHFR* 677C→T *CT* genotype (vs. CC) had little or no elevation in the rate of CVD (Table 4). Similarly, the *cSHMT*-CVD association varied according to the *MTHFR* genotype; i.e., the risk of CVD in men with the *cSHMT* 1420C→T *TT* genotype vs. men with the *cSHMT* 1420C→T *CC/CT* genotype was 0.4 (95% CI, 0.2–0.8), 1.6 (95% CI, 1.0–2.7), and 3.5 (95% CI, 0.9–14.1) among men with 0, 1, and 2 variant alleles at *MTHFR* 677C→T, respectively.

The homocysteine-genotype association was assessed considering each genotype in a separate model. The *cSHMT* 1420C→T genotype was associated with homocysteine; i.e., *TT* men had lower homocysteine than *CT/CC* men (9.9 vs. 10.7 μmol/L, respectively, $P = 0.09$). The *MTHFR* 677C→T *TT* genotype had a strong association ($P = 0.0014$) with plasma homocysteine; i.e., men with the *TT* genotype had higher homocysteine concentration than men with the *CT* and CC genotypes, respectively (11.8 vs. 10.4 μmol/L ($P = 0.0038$) and 10.4 μmol/L ($P = 0.0022$), respectively). Further models included both genotypes simultaneously and allowed for the gene-gene interaction; the difference in homocysteine concentration across the *MTHFR* 677C→T genotypes was much greater in men with the homozygote variant *cSHMT* 1420C→T genotype. In the *cSHMT* 1420C→T *TT* genotype stratum, men with the *MTHFR* 677C→T *TT* genotype had homocysteine concentrations 5.3 μmol/L greater than those of men with the *MTHFR* 677C→T CC genotype (14.8 vs. 9.5 μmol/L, respectively, $P = 0.02$). In the *cSHMT* 1420C→T *CT/CC* genotype stratum, men with the *MTHFR* 677C→T *TT* genotype had homocysteine concentrations 1.1 μmol/L greater than those of men with the *MTHFR* 677C→T CC genotype (11.7 vs. 10.6 μmol/L, respectively, $P = 0.13$).

To investigate whether the genotype-CVD association was mediated by plasma homocysteine, preliminary models confirmed that the same approximate results (before adjusting for homocysteine) were obtained in the reduced set of cases and controls with homocysteine data. Next, models were adjusted for homocysteine, and the findings did not differ from the unadjusted models (Tables 3 and 4). The effect of *MTHFR CT* and *TT* (vs. CC) genotype on CVD risk showed a strong dependency on *cSHMT* genotype, with effects of greatest magnitude observed among men with the *cSHMT TT* genotype. The larger SEs and wider CI in the adjusted models reflect the reduction in numbers, due to missing data on plasma homocysteine.

Other models explored the influence of potentially confounding variables. When risk factors for CVD were included in multivariate models (resulting in a reduction in the total number studied, from 1514 to 1418 in matched analyses), there was little or no change in the genotype-CVD model coefficients. Adjusted covariates included BMI, diastolic and systolic blood pressure, serum total cholesterol and triglycerides, current consumption of ≥2 drinks/d (yes/no), current smoking (yes/no), number of years smoking, and cumulative smoking dose (pack-years).

Lipid subfractions and dietary intake were considered as covariates; due to missing data, the inclusion of these variables in statistical models resulted in a moderate to substantial reduction in the number of observations. In the subset of cases and controls with available data, further adjusting for dietary folate; vitamins B-2 (i.e., riboflavin), B-6, and B-12; methionine; total energy intake; alcohol and coffee consumption; physical activity (energy spent in a week on average); and serum HDL cholesterol concentration had little or no effect on the regression coefficients for the genotype-CVD association.

TABLE 4

cSHMT 1420C→T genotype and CVD risk stratified by MTHFR 677C→T genotype, Normative Aging Study, 1961–1998

	cSHMT 1420C→T CC/CT		cSHMT 1420C→T TT	
MTHFR 677C→T	OR for CVD (95% CI)[1]	OR for CVD (95% CI)[2]	OR for CVD (95% CI)[1]	OR for CVD (95% CI)[2]
CT vs. CC	1.03 (0.8–1.2)	0.8 (0.5–1.1)	3.6 (1.7–7.8)	5.1 (1.5–17.7)
TT vs. CC	1.3 (0.9–1.8)	1.0 (0.6–1.6)	10.6 (2.5–46.0)	10.8 (1.3–88.5)

[1] Crude (unadjusted) OR, $n = 1514$ matched analysis, OR (95% CI) for the MTHFR 677-CVD association in each subgroup of cSHMT genotype calculated using the model parameters in Table 3, including 2 dichotomous variables, each indicating the effect of MTHFR 677 CT vs. CC and TT vs. CC, and their interaction with the dichotomous variable for the effect of cSHMT 1420 TT vs. CC/CT.
[2] Adjusted for homocysteine, $n = 987$ matched analysis, computed from combinations of the regression parameters in Table 3.

Given that dietary folate is expected to partially reverse the metabolic effects of the *MTHFR* polymorphism, we explored folate models further. The dietary supply of folate from food and supplements (assessed by the first available FFQ) had a weak inverse association with CVD risk. In analyses considering whether the gene-gene interaction (*cSHMT* by *MTHFR*) was modified by dietary folate, the 3-way interaction among *cSHMT*, *MTHFR*, and folate was not significant.

The NAS participants were white U.S. residents primarily of European descent, and ~70% of the men were European whites with identical paternal and maternal ethnic origins (721 of 1034 men); 85% of these 721 men were further categorized into 5 ethnic groups, and the prevalence of the *cSHMT* 1420C→T *TT* genotype ranged from 0 to 20% in the 5 groups. In models adjusted for ethnic group, there was little or no change in the magnitude of the genotype-CVD association.

DISCUSSION

A recently identified polymorphism in the gene encoding the cytoplasmic serine hydroxymethyltransferase enzyme (cSHMT), a key enzyme in the regulation of folate homeostasis, modifies the association of the *MTHFR* 677C→T genotype with the risk of CVD. This finding was supported by a similar pattern in plasma concentration of homocysteine. To the best of our knowledge, this is the first report of this gene-gene interaction in relation to CVD risk, and the first report of the relation between joint genotype status for *MTHFR* 677C→T and *cSHMT* 1420C→T and homocysteine, an established biological marker of 1-carbon metabolism.

The findings may be affected by other genetic variants that are in linkage disequilibrium with the *cSHMT* 1420C→T SNP. However, no other common polymorphisms were reported in the coding region of the *cSHMT* gene. The findings may be affected by population stratification; that is, the *cSHMT* polymorphism may be associated with other genetic and/or nongenetic causal factors that vary by ethnic group. However, including ethnicity as a covariate had little or no effect on the *cSHMT*-CVD association in these data. Moreover, the variation in allele frequency by ethnicity was within limits considered unlikely to cause confounding by population stratification (25).

The selection of the cases and/or case definition may influence the study findings. One-third of the incident CVD cases occurred before DNA sampling started, and the loss of these cases could bias the estimation of the gene-disease association. Among the cases without DNA samples, a greater proportion had early-onset CVD (age $<$ 60 y); in analyses limited to early-onset cases with DNA, the *cSHMT* 1420C→T by *MTHFR* 677C→T interaction was even stronger. Observed effect estimates in the full set of cases and controls may therefore underestimate the true association. An additional consideration is the definition of a case: the case definition was a mixture of endpoints (CVD only, stroke only, CVD and stroke). However, when stroke-only cases were excluded, the revised effect estimates were essentially unchanged.

The *cSHMT* polymorphism does not affect the catalytic activity or thermostability of the purified human cSHMT protein in vitro (unpublished data, Oppenheim, Szebenyi and Stover, Cornell University). Results from molecular modeling demonstrate that the 1420C→T polymorphism is not positioned to influence the enzyme active site, but rather is located on the exterior of the protein on a side chain that faces the bulk solvent (unpublished data, Oppenheim, Szebenyi, and Stover, Cornell University). The polymorphism results in a phenylalanine at amino acid position 474, which is located in a region of the protein shown to be involved in protein-protein interactions (unpublished data, Woller, Szebenyi, and Stover, Cornell University). Of the 3 postulated functions of cSHMT, the polymorphism is hypothesized to increase the amount of cSHMT available to bind with 5-methylTHF, thus increasing the sequestering of 5-methylTHF and decreasing *S*-adenosyl methionine synthesis. This explanation is consistent with our observation of a greater effect of the *MTHFR* polymorphism in men with the *cSHMT* polymorphism, and highest levels of homocysteine in men homozygous for both polymorphisms.

The *MTHFR* 677C→T variant disrupts the riboflavin-binding site of the enzyme, and the diminished enzyme activity associated with the polymorphism is stabilized by an adequate supply of intracellular folate (26,27). Intracellular folate status is affected by the dietary intake of folate (1). The net effect of the sequestering of 5-methylTHF by cSHMT may also be affected by folate supply. There was no statistical evidence for a 3-way interaction; however, the elevated risk associated with having both *cSHMT* 1420C→T *TT* and *MTHFR* 677C→T *CT*/*TT* genotypes was attenuated when the analysis was limited to men with intakes of folate above the median. There was no evidence of further effect modification by other methylation-related dietary factors including vitamins B-2 (riboflavin), B-6, and B-12 (cofactors of folate-regulating enzymes, MTHFR, cSHMT, and methionine synthase, respectively).

Although the functional consequences of the *cSHMT* 1420C→T polymorphism are still under investigation, studies demonstrated effects on homocysteine (6), and leukemia risk (8), but no association with colorectal cancer (9). The study reported herein investigated the association between a novel polymorphism in *cSHMT* and the risk of CVD. We observed a gene-gene interaction such that the association of *MTHFR* 677C→T and CVD varied by the *cSHMT* genotype. The increased risk of CVD associated with MTHFR 677C→T *CT* and *TT* genotypes was of greater magnitude among men with the *cSHMT* 1420C→T *TT* genotype. The genotype-CVD association was confirmed by the genotype-homocysteine association, although the association of genotype was not mediated by homocysteine in these data. The exact role of homocysteine as a causal factor vs. risk marker remains to be determined. Further studies are required to confirm these findings, and to better understand the relation of genotype to biological markers of folate-dependent methylation, including homocysteine, SAM, and SAH; this will advance our understanding of the biological mechanism underlying these findings.

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